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Chris Proudfoot[1] and Christine Burkard[1,*]

1 The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, EH25 9RG Midlothian, United Kingdom.

* Corresponding author

E-mail: Christine.burkard@roslin.ed.ac.uk

Abstract (250 words)

One of the major burdens on the livestock industry is loss of animals and decrease of production efficiency due to disease. Advances in sequencing technology and genome editing techniques provide the unique opportunity to generate animals with improved traits. In this review we discuss the techniques currently applied to genetic manipulation of livestock species and the efforts in making animals disease resistant or resilient.

Abbreviations list

ASFV	African swine fever virus
BSE	Bovine spongiform encephalopathy
bTB	Bovine Tuberculosis
Cas	CRISPR-associated gene
CPI	Cytoplasmic microinjection
CRISPR	Clustered regularly interspaced short palindromic repeats
DBD	DNA binding domain
DSB	Double strand break
GeCKO	Genome-wide CRISPR knock-out
GWAS	Genome-wide association study
HDR	Homology directed repair
HE	Homing endonuclease
iPSC	induced pluripotent stem cell
IVF	*In vitro* fertilisation
KO	Knockout
NHEJ	Non-homologous end joining
PGC	Primordial germ cell
PNI	Pronuclear microinjection
PrP	Prion protein
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
SCNT	Somatic cell nuclear transfer
siRNA	small interfering RNA
SMGT	Sperm-mediated gene transfer
SSC	Spermatogonial stem cells
ssRNA	single-stranded RNA
TALE	Transcription activator-like effector

TALEN Transcription activator-like effector nuclease

ZF Zinc finger

ZFN Zinc finger nuclease

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Introduction

With the world population predicted to reach almost 10 billion by 2050 there are a number of challenges in sustainable management of finite resources. The rising demand for food requires improved productivity of agricultural systems. One of the major burdens on the livestock industry is loss of animals and decrease of production efficiency due to disease. Furthermore, it is important to improve the health and welfare of animals by reducing and preferably preventing the effects of disease. Advances in sequencing technology and genome editing techniques provide the unique opportunity to generate animals with improved traits. In this review we will discuss the techniques currently applied to genetic manipulation of livestock species and the efforts in making animals disease resistant or resilient.

The tools

In 1982 Plamiter and Brinster (1) set the stage for sequential advances in our ability to modify and improve mammalian genomes for desirable traits. Whereas previous work by others showed that foreign DNA fragments could be integrated into the genome of embryos by pronuclear microinjection (PNI), their work demonstrated a functional application; introduction of a growth hormone gene into mouse embryos resulted in rapid growth of the animals. Beyond utilizing cell-based approaches, early genome modification was restricted to the injection of plasmids or gene fragments into the pronucleus of embryos. More efficient integration of foreign DNA fragments into the target genome was subsequently achieved using transposons or retroviral vectors (2, 3). Early specific edits in cells could be achieved with homing endonucleases (HE), natural meganucleases, which introduce double strand breaks (DSBs) at target recognition sites of 14-40bp (4). However, engineering of HEs has been challenging and they are prone to off-target cutting, wherefore there are currently still very few *in vivo* applications (5). The development of the zinc finger nuclease (ZFN) (6), the first genome editing tool, increased the repertoire of programmed modifications, allowing precise cutting and repair to any target genome. The intervening two decades have borne witness to the continued development of the editing toolbox, with improvements in adaptability and efficiency, coupled with reduced costs and facile in-house assembly platforms, resulting in an almost exponential uptake of the technology in the last five years (Figure 1).

Zinc Fingers (ZFs) are amongst the most well-characterised protein DNA binding domains (DBDs) found in nature. Each ZF binds a triplet of nucleotides, with synthetic arrays of ZFs constructed to improve specificity to a desired target sequence (typically 9-18 bases). ZFNs are chimeric enzymes created by

fusing a modular ZF array to the nuclease domain of the restriction enzyme FokI. This nuclease domain has no innate sequence specificity, with target site delineated by the ZF array. The FokI nuclease domain requires dimerization to function, so paired ZFNs are typically employed to generate a targeted DNA DSB, further increasing site specificity. Unfortunately, the design of ZFNs remains a complicated and technically challenging process. Only a small number of companies have the expertise required to produce reliable ZFNs, and, as a result, these reagents have remained relatively expensive, curbing their wider use.

Transcription activator-like effectors (TALEs) are a second group of naturally occurring proteins containing DBDs. Produced by proteobacteria of the genus *Xanthomonas*, TALEs bind host DNA and thereby alter the transcriptional profile of infected cells. The DNA binding portion of each TALE is composed of a repeated modular array, with each module having sequence preference for a single DNA base (7). This simple 1-to-1, module-to-base, relationship makes design of functional synthetic DBDs straight forward, and kits can be purchased to allow their assembly in a standard molecular biology lab in less than two weeks. Typically designed to recognise 12-20 bases, arrays are fused with FokI to give a TALE nuclease (TALEN), and, as with ZFNs, these are employed in pairs to allow FokI dimerization and increase specificity. Two sequential modifications to the FokI domain further reduced the potential for off-target cutting by both TALENs and ZFNs; conversion of the homodimer into an obligate heterodimer, and conversion from a nuclease to a nickase by mutation of a catalytic domain (8). However, while TALENs were certainly more widely utilised than ZFNs, they have since been superseded by the most recent tool.

Developed from an innate bacterial antiviral mechanism, the latest addition to the genome editor toolbox is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system. With target specificity directed by a short single-stranded RNA (ssRNA) molecule(s), this represents a departure from the preceding genome editors that utilise a protein-based DBD. As with the FokI nuclease domain, Cas nucleases lack innate sequence specificity but are instead guided to their target site by Watson-Crick base pairing between their complexed ssRNA and the cognate DNA sequence. This constitutes a major advantage for this tool; the Cas nuclease is not covalently fused to a DBD so the same protein can be utilised to target multiple different target sites simply by combining it with different combinations of ssRNA. Furthermore, while all of the reagents required can be produced in almost any molecular biology lab, both the Cas nuclease and the ssRNA molecules can also be purchased from multiple vendors. The ease with which reagents can be designed, coupled with economical availability, has resulted in huge uptake of this tool and an explosion of publications in this field. (9, 10)

The possibilities

Genome editors can break DNA at specific target sites; it is through the subsequent repair of these breaks that scientists can introduce desired changes at the target locus. Most cell types preferentially utilise the non-homologous end-joining (NHEJ) pathway, an error-prone process that typically results in small insertions or deletions at the site of repair. By creating a DNA break in the coding sequence of a gene, this form of repair often generates a frameshift mutation and thereby truncation of the encoded protein or functional gene knockout (KO). Alternatively, by flooding the target cell with a DNA repair template, it is possible to trigger the homology-directed repair (HDR) pathway. This allows the introduction of precise sequence changes proximal to the cut site, ranging from single base changes to the insertion of transgenes. Researchers commonly use synthetic single stranded oligodeoxynucleotides to introduce small changes, or plasmid/dsDNA templates for larger insertions. Finally, by creating two simultaneous breaks on a chromosome the intervening DNA sequence can be deleted; this approach can be used to alter transcriptional profiles by removing regulatory elements, or to delete exons thereby removing protein domains while leaving the remaining reading frame intact. (11)

As a result of the evolution of this technology and a greater understanding of how to harness its potential we are now able to introduce extremely precise changes to the genome with greater accuracy and efficiency than has ever been possible. We are now at a stage where we are limited more by our imagination than the technology available.

The industry

The long history of livestock domestication has relied on the sequential selection of animals based on desirable traits, with generational improvements in their ability to thrive in the varied habitats occupied by the communities farming them. Traditionally, selective breeding focused on handling and productivity traits, such as docility, feed conversion, and fertility, with modern breeding goals incorporating animal health and welfare. Selecting for disease resistance and resilience is not only important from an animal health and welfare perspective but has significant economic impacts. For instance, it is estimated that endemic diseases incur added costs of €30-40 per slaughter pig in the European Union, adding to up to €10.5bn per year ((12), EU28 2016 (13)).

Livestock breeding companies incorporate genetic improvement into their programmes by assigning values to various traits of individual animals and incorporating only those with the highest overall merit into their nucleus herd. Advances in affordable genotyping tools allow direct linkage between physiological characteristics and genome-wide association studies (GWAS) (14), resulting in increasingly efficient and productive breeding populations. Compilation of phenotypic information is relatively straightforward when traits can be measured accurately under normal husbandry conditions. By contrast, disease susceptibility, or lack thereof, is a trait that is difficult to quantitatively assess as exposure to pathogens within a population of animals is rarely uniform and the deliberate exposure of

large numbers of animals under experimental conditions is both ethically questionable and very expensive (15). Even if such variance could be readily identified, the merit gains achieved by recent breeding programmes may prove a barrier to propagation within a nucleus herd; the allelic variant might be present in low abundance, recessive, or associated with animals that would otherwise be considered of low merit. In this scenario genome editing offers an opportunity to contribute to the natural breeding process, introducing newly identified genetic features into the progeny of elite nucleus animals without negatively impacting other highly desirable traits (16). Such an approach could also contribute to genetic improvement if relevant polymorphisms were identified in related breeds or even other species (Figure 2). As such, genome editors have great potential in allowing the introduction of novel traits that improve animal welfare, increase production, reduce food waste in the production chain, improve food security, and contribute to the economic security of small holder farmers.

The animals

Editing in **cattle** poses a significant challenge due to cost, small number of offspring, and long generation time (9 months gestation, 12-18 months to reach sexual maturity). As a consequence, there is significant pressure for editing techniques to be highly efficient to ensure intended offspring. While somatic cell nuclear transfer (SCNT) of confirmed edited cells is often the preferred option (17, 18) cytoplasmic microinjection (CPI) and PNI into *in vitro* fertilised oocytes have also been employed (19). While generation of chimeras by microinjection of edited induced pluripotent stem cells (iPSCs) into blastocysts has been demonstrated in multiple papers, so far no germline transmission was reported (20, 21). A potential future editing technique in cattle may also be the editing of spermatogonial stem cells (SSCs). Long-term cultivation methods have been recently published and advances in transplantation of these cells and sterile recipients could provide a promising avenue for generating genome edited cattle (22, 23).

Editing **goats and sheep** is less restricted than cattle; they are smaller, cheaper, and produce more offspring and, with gestation times of 5 months and sexual maturity at 6-8 months, the generation times are significantly lower. Oocytes can be collected from abattoir samples or by laparoscopic ovum pick-up, with *in vitro* fertilization (IVF) and microinjection of zygotes (24). In all ruminants blastocysts can be re-implanted into a recipient, allowing testing embryo viability and genotype prior to the implantation. Alternatively, small ruminants have also been generated by SCNT (25-27) and goats by sperm-mediated gene transfer (SMGT) (28). The generation of chimeric embryos from (non-edited) iPSCs has been demonstrated in sheep (29), whilst in goats the generation of iPSCs has been reported, no editing or chimeric integration has been demonstrated yet (30, 31).

Pigs have several advantages over ruminants for genome modification; they have large litter sizes, a short gestation of less than 4 months, and can reach sexual maturity at 5-6 months. While many groups have successfully used SCNT to generate modified pigs, microinjection and transfer of zygotes is an

efficient alternative. As pigs are a multiparous species that self-limit the number of embryos they carry to term, an excess of manipulated zygotes can be transferred to each recipient to improve pregnancy rates. Zygotes are generally harvested from donor animals as polyspermy associated with IVF remains a significant issue (32). Genome modified animals have been produced using a variety of techniques, including SCNT and PNI and CPI of zygotes (comprehensive lists of modified animals can be found (33, 34)). Germline transmission has been demonstrated in chimeric animals generated from iPSCs (35, 36).A variety of cultivation methods for SSCs have been described in pigs, whereas long-term cultivation still remains a challenge (reviewed in (37)). Furthermore, genome edited, sterile recipients for the transfer of (edited) SSCs have been described in pigs (38).

Editing in **chicken** has proven challenging compared to mammals due to significant differences in reproductive physiology. The germinal disk of a laid egg consists of approximately 50,000 cells leaving no clear route for efficient modification. Editing in chicken currently relies on primordial germ cells (PGCs) that are edited *in vitro* and transferred into recipient embryos. PGCs can be isolated, cultivated and genetically modified whilst maintaining their PGC status (39, 40). Transfer of PGCs to the blood stream of recipient embryos results colonisation of the developing gonad and subsequent germline transmission (41). With PGCs it is now possible to manipulate the genome of the chicken in culture and to use those cells to establish an edited chicken line. Furthermore, chicken PGCs may be modified by microinjection of transfection reagents and transposons into the blood stream of embryos to generate germline-modified animals (42).

Whilst there has been a long history in management and breeding of the aforementioned animals, there are in fact numerous other livestock species that confer significant economic impact to the agricultural sector.

The global consumption of **farmed fish** has recently overtaken beef and is the fastest growing animal protein production sector. Whilst selective breeding in fish is utilised for many species, targeted genome modification is in its infancy (43). Fish lay large numbers of eggs that are externally fertilised and thereby readily accessible for genetic manipulation. Early examples of transgenic fish are growth hormone transgenic salmon and Nile tilapia (44, 45). Genome edited salmon have been produced using CRISPR/Cas9 to induce albinism (46) or infertility (47). Both of these traits are of potential utility for Salmon breeders as albinism can serve as a visual editing indicator and sterility would prevent interbreeding of edited with wild salmon. Catfish were edited for enhanced growth by using CRISPR/Cas9 to knock out the myostatin gene (48). No genome editing for disease resistance has yet been reported in fish, however multiple GWAS studies are currently being conducted to identify such targets. A potential target region has been identified in salmon conferring resistance against pancreatic necrosis (49).

FAO lists **honeybees** as livestock as they are integral to many agricultural practices. Global impact of honeybees as pollinators in crop production is significant (50, 51). Selective breeding in bees is employed to select for hygienic, low disease burden colonies (52, 53). Edited or transgenic bees can be generated by microinjection of embryos (54, 55). As has been the case in many agricultural species early work involved the introduction of fluorescence markers into the genome (54). Application of genome editors thus far has been to identify gene function rather than to address disease resistance (55). With improvements in the understanding of the bee genome and more detailed association studies it is anticipated that genome editing for disease resistance in bees is in the future (56).

The diseases

Mastitis has a huge impact in the dairy industries. In the US it is the most common disease in dairy cattle resulting in estimated annual losses of $2bn. Globally, small ruminants also play an important role in the dairy industries with mastitis conferring a significant economic burden. *Staphylococcus aureus* is the most common pathogen to cause mastitis and there is a very low natural heritability of resilience to infection. In ruminants a number of similar transgenic strategies have been employed by generating animals producing enzymes inhibiting the growth of bacteria in the mammary gland. In cattle the antibiotic lysostaphin was introduced by SCNT resulting in secreted protein in their milk, capable of killing *S.aureus* (57). The milk from goats expressing human lysozyme was shown to inhibit the growth of mastitis-causing bacteria and *Pseudomonas fragi*, responsible for the cold-spoilage of milk (58, 59). Importantly, the growth of *L. lactis*, required for the making of processed dairy products, such as cheese, was not inhibited.

Misfolding of the prion protein (PrP) is associated with neurogenerative diseases in many mammals. The accumulation of misfolded PrP plaques results in bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep and goats. A number of different groups have knocked out the PrP gene as a strategy to circumvent such diseases. While a transgenic approach has been used to achieve this goal, application of genome editors could be used streamline this process. In addition to value to the agricultural sector, interest in PrP KO livestock extends to biopharmaceuticals, as this is considered an appropriate safety measure of products destined for human applications (60-63).

Early efforts are ongoing to make ruminants resilient to *Mannheimia (Pasteurella) haemolytica* infection, which causes epizootic pneumonia (shipping fever) and may also contribute to enzootic pneumonia in calves and lambs as well as peritonitis in sheep. The pathogen can produce a cytotoxic leukotoxin, which is largely responsible for the pathogenicity of the bacteria. In ruminants the leukotoxin binds to the uncleaved signal peptide of the CD18 protein present on the cell surface leukocytes (64). In other species, including mouse and human, the mature CD18 lacks the signal peptide as a result of proteolytic processing. Based on the human sequence, ZFNs were used to introduce a single amino acid change.

Leukocytes from the resultant foetuses were resistant to cytotoxicity associated with *M.haemolytica* leukotoxin (65).

Bovine tuberculosis (bTB) has a direct effect on productivity in cattle and buffalo, impacts international trade, and poses a significant human health risk. Polymorphisms in the NRAMP1 gene, also known as SLC11A1, in cattle have been associated with varying levels of resilience to bTB infection (66). CRISPR/Cas9 was used for targeted insertion of an NRAMP1 variant associated with resilience to bTB infection into the cattle genome. *Ex vivo* challenge of peripheral blood monocytes showed reduced pathogen growth in exogenous NRAMP1 expressing cells. An *in vivo* study in the transgenic animals reported diminished interferon response to TB infection but did not assess pathogen burden (67).

African Swine Fever Virus (ASFV) is a disease endemic to huge swathes of sub-Saharan Africa. Native suid hosts, including the warthog, are resilient to the infection, while domestic pigs develop a lethal haemorrhagic fever. Species-specific variation of the *RELA*, a component of the transcription factor NF-κb, between native and domestic suids were postulated to underlie this host genetic variance (68). Using a ZFN pair with a plasmid template for HDR, researchers converted the encoded domestic pig protein sequence to the warthog equivalent (69). Data to show resilience of the animals to ASFV infection has yet to be reported. It is important to differentiate between disease resistance, the ability of an animal to suppress the establishment and/or development of an infection, and disease resilience where an infected host manages to maintain an acceptable level of productivity despite challenge pressure (70). Should these pigs prove to be resilient to ASFV infection it is likely that their use may not be permitted in many jurisdictions, since they could act as reservoirs of infection. However, in environments where the disease is endemic use of such animals could be beneficial.

The most economically important pig disease worldwide is porcine reproductive and respiratory syndrome (PRRS). *In vitro* experiments showed that entry of the causative agent of the disease, PRRS virus (PRRSV), into host cells relies on two proteins, CD163 and CD169 (71). It was further demonstrated that subdomain 5 of CD163 was essential for PRRSV entry (72). Surprisingly, SCNT with fibroblasts lacking CD169 resulted in pigs that were not resistant to PRRSV infection (73). Functional CD163 KO animals were generated using a CRISPR/Cas9 to induce a NHEJ-mediated premature stop codon (74). The CD163 KO animals were shown to be resistant to PRRSV infection both *in vitro* and *in vivo* (75). CD163 has a wide variety of important biological functions in inflammation and immune response. To retain these functions precise deletion of only CD163 subdomain 5 has been carried out. Subdomain 5 is encoded by exon 7, which was excised from the genome using CPI of two guide RNAs targeting the flanking intronic sequence. Cells from the resulting animals are resistant to PRRSV infection and maintain their biological function (76).

Reactivation of endogenous retroviruses is a potential barrier to the use of livestock as tissue and organ donors. Genome editors have been used to permanently inactivate porcine endogenous retrovirus in pigs presenting a potential solution to this human health threat (77).

Avian influenza poses a significant threat to the global poultry industry and to human health, as zoonotic transmission is frequently observed. The control of outbreaks requires the culling of infected and neighbouring flocks and the implementation of strict biosecurity measure to prevent the further spread of the virus. Transgenic chickens were generated by microinjection of eggs with retrovirus to incorporate a small decoy RNA fragment under a U6 promoter into the chicken genome (78). The decoy RNA fragment expressed in chickens interferes with the formation of infectious influenza particles, thereby preventing spread to co-housed birds. This approach has yet to be evaluated in other species susceptible to influenza.

Discussion and Outlook

Application of genome editors allows easy-to use, targeted strategies for genome modification in livestock. To improve disease resistance traits, editing targets are identified by investigation of in vitro host-pathogen interactions, species variation, or GWAS studies (Figure 3). The field of genome editors is fast evolving and sequential improvements coupled with a better understanding of DSB repair mechanisms will inevitably result in an expanded range of editing opportunities. Advances in delivery techniques, such as editing gametes or spermatogonial, embryonic, or induced pluripotent stem cells, will streamline the production of edited animals and make it applicable to a wider range of species. Generating disease resistant animals will not only help to feed the world but also improve animal welfare and aid in the reduction of antimicrobial use.

Most of the examples discussed in this review are still at early stages and integration of genome edited animals into highly productive elite breeding lines will take time. Furthermore, approval of edited animals for human consumption relies on national and multi-national legislation, which is currently at early stages. And in the end, also the consumer will decide on the success of genome edited animals in livestock production.

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Figure Legends

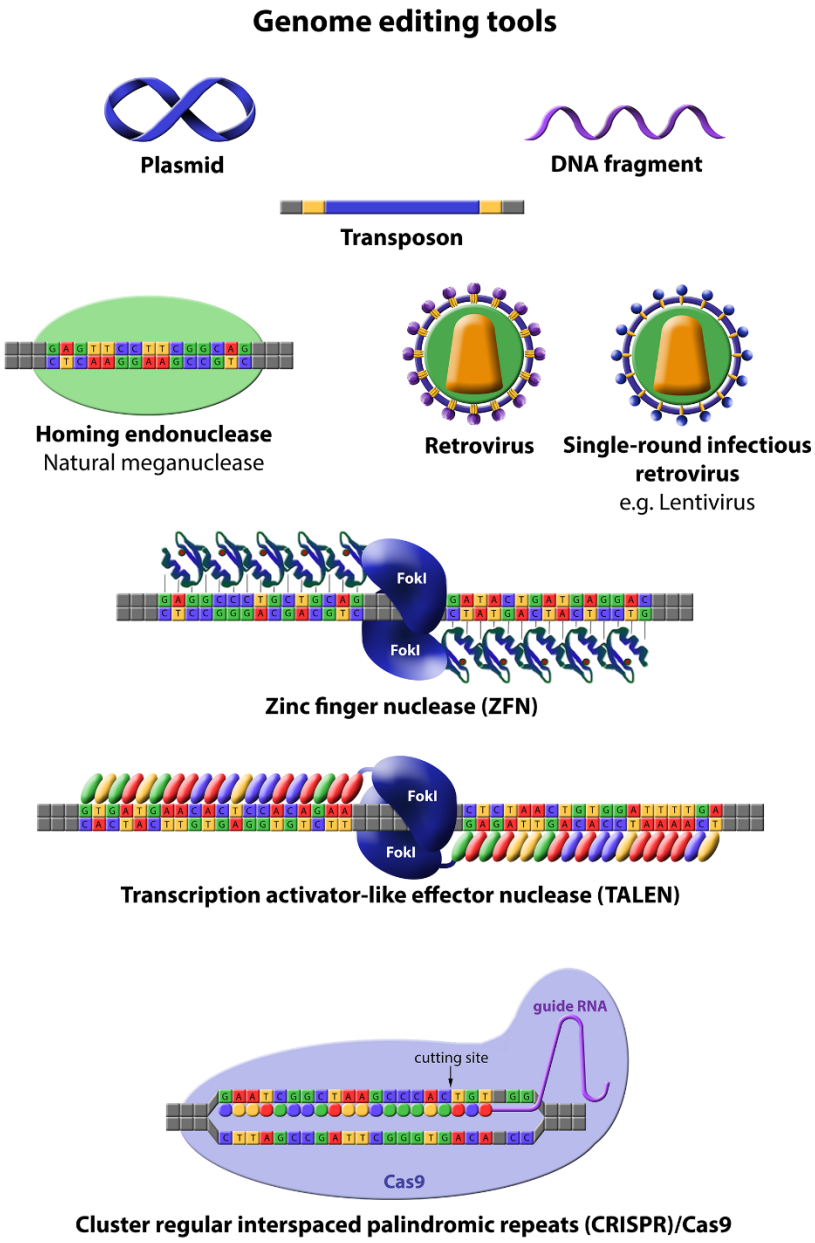

Figure 1: Genome editing tools. By transfection of plasmids, transposons or DNA fragments and random or homology-directed integration foreign DNA fragments or modifications may be introduced into a target genome. Retroviruses or single-round infectious retroviruses may be used to integrate foreign genes at random sites into the target genome. Homing endonucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 are genome editors relying on enzymatic activity to introduce a targeted double strand break in the genome. Repair of these double strand breaks can lead to non-homologous end joining events, resulting in insertion or deletions. A combination of genome editors with DNA fragments, plasmids or transposons can be used to enhance the efficiency of homology-directed repair to integrate foreign genes or modify single base pairs at a specific locus in the genome.

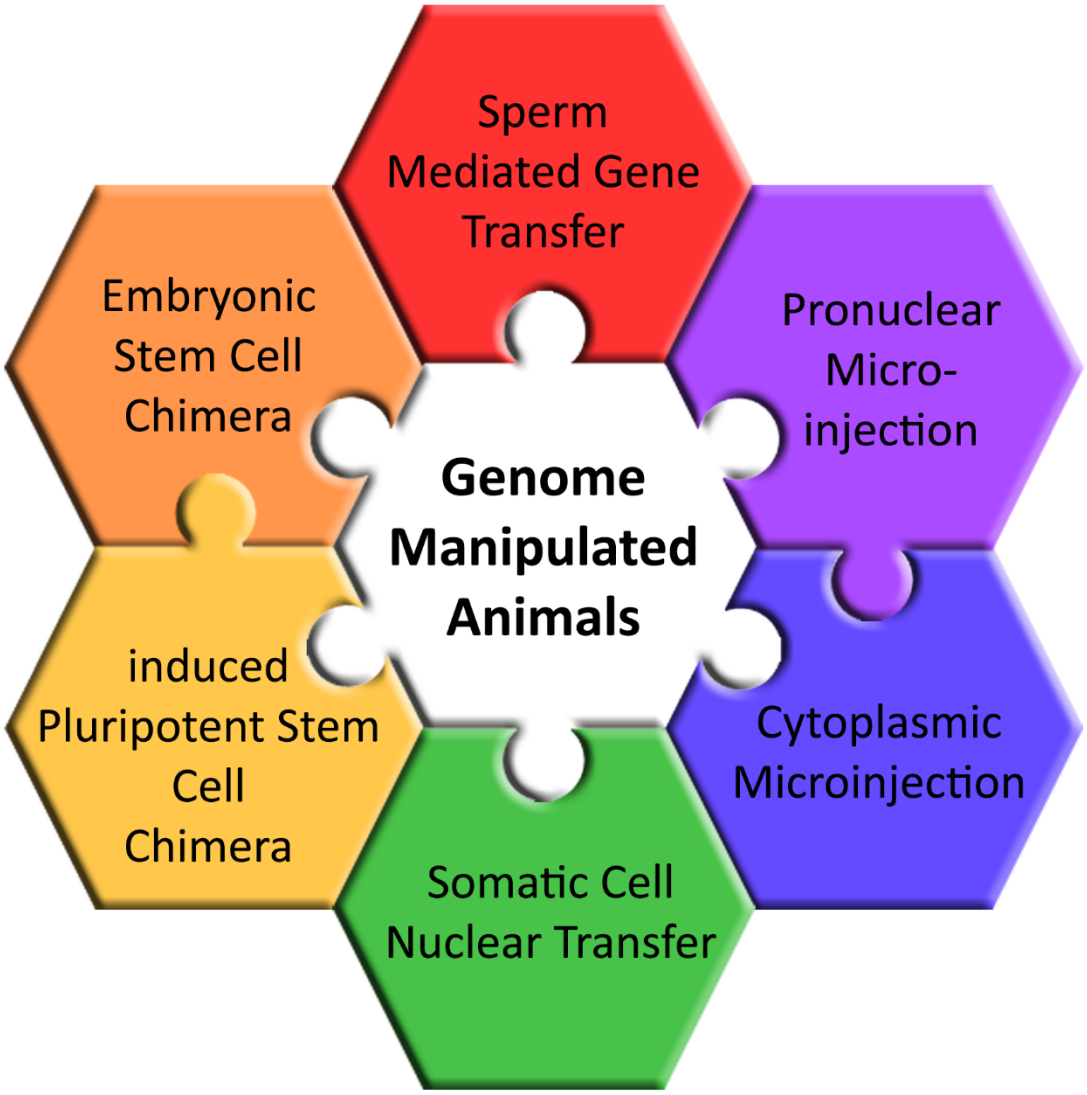

Figure 2: Techniques to generate genome-manipulated animals. Using genome editing tools a variety of techniques are available for the generation of genome edited animals. Sperm-mediated gene transfer (SMGT) is used for the delivery of genome editing reagents to the zygote. Furthermore, spermatogonial stem cell (SSC) or primordial germ cell (PGC) manipulation offer new avenues to generate edited animals. Pronuclear and cytoplasmic injection of genome editors into zygotes and subsequent transfer to surrogates or maturation in incubators prior to transfer may be used to generate genome-manipulated animals. Somatic cell nuclear transfer allows the selection of specific edits in somatic cells prior to nuclear transfer to surrogates or maturation in incubators. The injection of edited embryonic or induced pluripotent stem cells into blastocysts can also be used to generate chimeric edited animals.

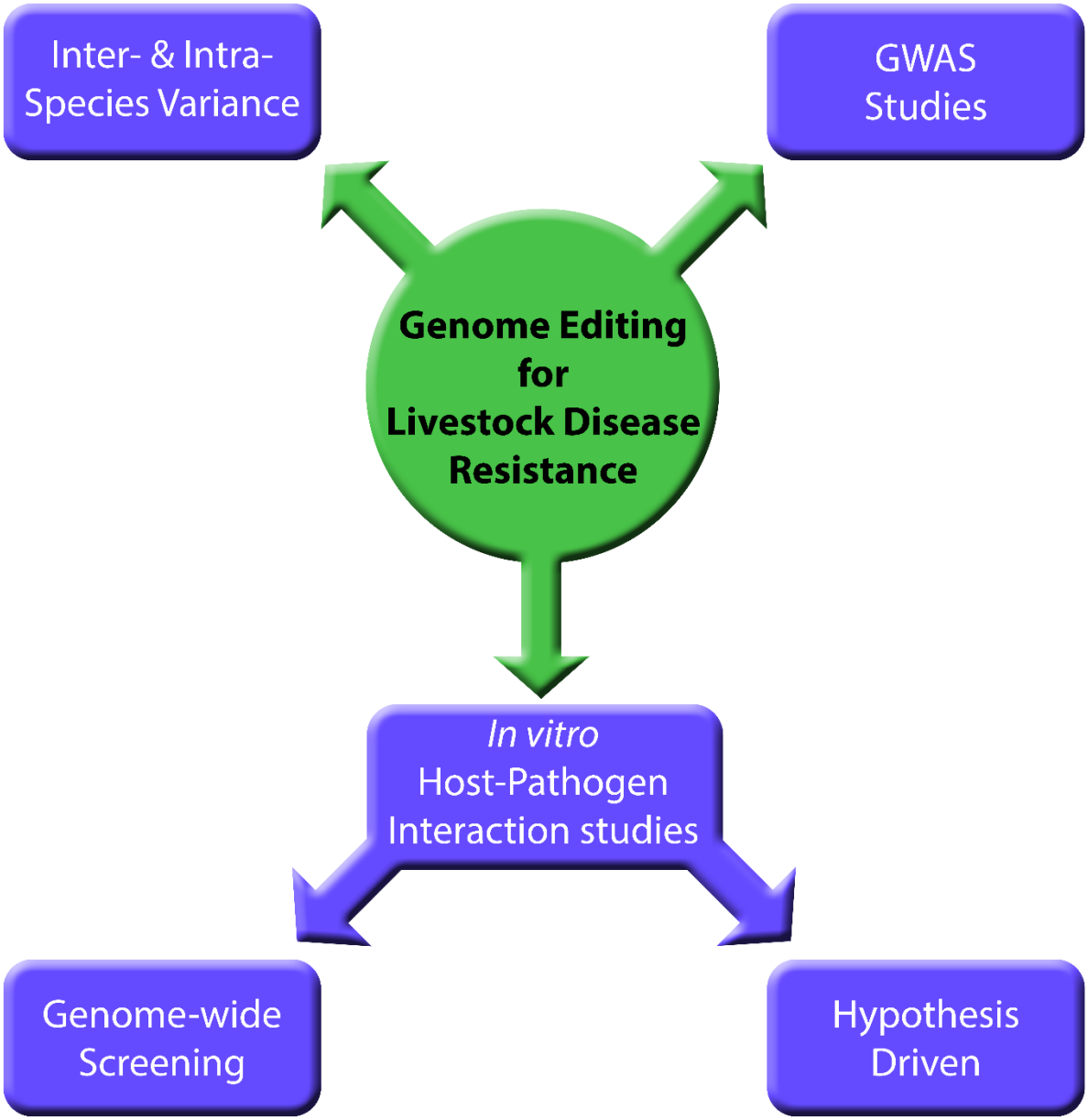

Figure 3: Research paths to identify editing targets for disease resistance. Identification of sequence variation associated with susceptibility to disease state allows for the identification of potential editing targets. Genome-wide association studies (GWAS) allow high throughput comparisons between large numbers of animals of the same species. Hypothesis-driven analysis of specific gene sequences can be used to identify species variance associated with disease resistance. In a laboratory setting *in vitro* host-pathogen interaction studies can be used to identify pathways amenable to interventions. Genome-wide screening methods, such as small-interfering RNA (siRNA) or genome-scale CRISPR knock-out (GeCKO), allow for the identification of a large number of proteins required for pathogen replication. Hypothesis-driven strategies rely on a prior knowledge of specific host-pathogen interactions.